Cat. # T100A/B

For Research Use

TaKaRa

RetroNectin®

(Recombinant Human Fibronectin Fragment)

Product Manual

v201705

Table of Contents

I. Description

RetroNectin reagent is a recombinant human fibronectin fragment (rFN-CH-296) composed of three functional domains: the cell-binding domain (C-domain), heparin-binding domain (H-domain), and CS-1 sequence. The fragment enhances retroviral-mediated gene transduction by aiding the co-localization of target cells and virions. Specifically, virus particles bind RetroNectin reagent via interaction with the H-domain, and target cells bind mainly through the interaction of cell surface integrin receptor VLA-5 and VLA-4 with the fibronectin C-domain and CS-1 site, respectively. By facilitating close proximity, RetroNectin reagent can enhance retroviral-mediated gene transfer to target cells expressing integrin receptors VLA-4 and/or VLA-5.*1

There are two RetroNectin-mediated infection protocols: the supernatant (SN) infection method and the RetroNectin-bound virus (RBV) infection method.[5],*2 With the SN infection method, cells are mixed with virus supernatant and loaded on a RetroNectin-coated plate. In the RBV method, the retrovirus is first bound to the RetroNectin-coated plate, and cells are added after removing the retrovirus supernatant. Removal of the supernatant reduces inhibitory molecules (e.g., molecules secreted from the producer cells such as proteoglycans and/or viral envelope proteins) that can reduce the efficiency of viral-mediated gene transduction.

*1 RetroNectin can also enhance lentiviral-mediated gene transfer.

*2 Both methods can be used for efficient gene transduction. Although the RBV infection method is widely applicable, some modification might be required depending on the target cells, vectors, and/or target genes.

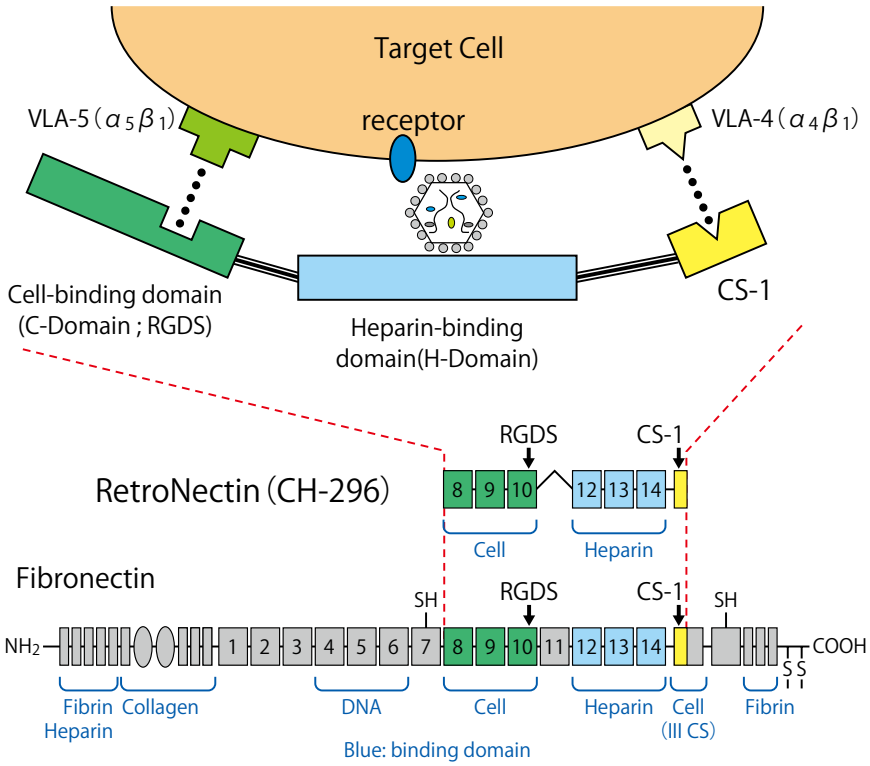

Figure 1. The hypothesized mechanism of RetroNectin-mediated transduction. The cell binds to the CS-1 site via VLA-4, and to the C-domain via VLA-5. The viral particle can bind to the H-domain of RetroNectin. These interactions increase the localized concentrations of cells and viral particles, an effect that is thought to enhance gene transduction.

II. Components

RetroNectin (Recombinant Human Fibronectin Fragment)	0.5 mg (Cat. #T100A)
RetroNectin (Recombinant Human Fibronectin Fragment)	2.5 mg (Cat. #T100B)

RetroNectin is provided as a sterile 1 mg/ml solution.
[Form] Sterile solution containing 12.5 mM sodium citrate (pH 6.2) and 1.25% sucrose

III. Storage

-20℃

Caution: Freezing and thawing can be repeated up to 10 times.
Do not mix the solution vigorously. Do not vortex.

IV. Materials Required but not Provided

[Equipment]

- Non-treated tissue culture plates or dishes
- Electric pipetter
- Pipetter
- Sterile pipettes
- Sterile tips with filters
- Safety cabinet or clean workstation
- Microscope
- CO_2 incubator
- Microplate centrifuge

[Reagents]

- Sterile PBS (-)
- HBSS/Hepes (Hank's Balanced Salt Solution supplemented with 2.5% (v/v) 1 M Hepes)
- 2% bovine serum albumin (BSA Fraction V)/PBS Solution

V. Protocol

【 1. Preparation of RetroNectin-Coated Plates 】

Coat a plate using 20 - 100 μg/ml RetroNectin with a volume corresponding to 4 - 20 μg/cm^2 plate area.

(1) Prior to coating, prepare a RetroNectin solution (20 - 100 μg/ml*1) by diluting with sterile PBS.

*1 Example: calculating the amount of RetroNectin reagent
When 2.25 ml of RetroNectin solution at a concentration of 20 μg/ml is placed into a 35 mm diameter dish (9 cm^2), the concentration used for coating is 5 μg/cm^2.

Note: To avoid loss of RetroNectin fragment, do not filter-sterilize RetroNectin solution diluted with PBS.

(2) Dispense an appropriate volume*2 of sterile RetroNectin solution into each plate and allow the plate to stand for 2 hours at room temperature or at 4℃ overnight.

*2 Dispense 0.5 ml into each well of a 24-well plate or 2 ml into each well of a 6-well plate.

Note: Non-treated, cell culture-grade tissue culture plates or dishes should be used in this step.

(3) Remove the RetroNectin solution and then block with an appropriate volume*3 of sterile 2% bovine serum albumin (BSA, Fraction V) in PBS. Allow the plate to stand at room temperature for 30 minutes.

*3 Use 0.5 ml for each well of a 24-well plate or 2 ml for each well of a 6-well plate.

(4) Remove the BSA solution, and wash the plate once with an appropriate volume of HBSS/Hepes or PBS. After removing the wash solution, the plate is ready for use. The RetroNectin-coated plate can be sealed with Parafilm and stored at 4℃ for up to one week.

【 2. Gene Transduction 】

There are two methods of gene transduction using RetroNectin reagent: RetroNectin-bound virus (RBV) infection method (Section A) and supernatant (SN) infection method (Section B). In the RBV infection method, retroviral particles are first bound to the plate coated with RetroNectin reagent, and the target cells are added after removing the virus supernatant. In the SN infection method, the virus solution and target cells are mixed and then added to the RetroNectin-coated plate.
When virus solution is directly used for virus infection without purification, the gene transduction efficiency may be reduced because of contaminating molecules that inhibit infection. In such cases, the RBV infection method is recommended. With this method, inhibitory molecules can be removed by binding viruses to RetroNectin reagent and removing the supernatant.

Note: Using a transient production system, such as the Retrovirus Packaging Kit Eco (Cat. #6160)*/Retrovirus Packaging Kit Ampho (Cat. #6161)* and the pDON-AI-2 Neo DNA (Cat. #3653)/pDON-AI-2 DNA (Cat. #3654), retroviral vectors can be prepared in one week.

* Not available in all geographic locations. Check for availability in your region.

A. RetroNectin-Bound Virus (RBV) Infection Method

A-1. Preparation of virus-bound plate without centrifugation

1. Add retrovirus supernatant at 125 - 250 μl/cm^2 to a RetroNectin-coated plate or dish.
2. Incubate for 4 to 6 hours at 32℃ or 37℃ in a 5% CO_2 incubator to promote binding of the virus particles with RetroNectin reagent.
3. Discard the supernatant, but do not allow the plate to dry. Wash the plate with an appropriate volume of PBS or PBS containing 0.1 - 2% albumin (BSA or HSA). After washing, perform infection according to A-3.

A-2. Preparation of virus binding plate by centrifugation

If the virus titer is high enough, binding of viruses with RetroNectin reagent can be accomplished without centrifugation as described in A-1.
However, if the titer is low, or if you require higher gene transduction efficiency, binding the virus by centrifugation is preferable. With this method the time required to bind the retrovirus is significantly less (2 hours versus 4 - 6 hours for the non-centrifugation method). A plate, such as a non-treated cell culture plate, that can tolerate centrifugation at 1,000 - 2,000*g* for 2 hours at 32℃ is required for this method. In addition, please note there is a possibility of aerosol formation.

1. Add the retrovirus stock solution or diluted solution at 125 - 500 μl/cm^2 to the RetroNectin-coated plate.

   Note: The volume that can be added to the plate varies. For a 6-well plate, the upper limit is 3 ml (320 μl/cm^2). In this method, some infection-inhibiting molecules may not be removed by washing with PBS. For this reason the efficiency of gene transduction might be reduced. In such a case, it is recommended that the virus stock solution be used after dilution with growth medium. Optimization is required to determine the suitable dilution rate.

2. Place the plate in a centrifuge pre-warmed to 32℃ and centrifuge for 2 hours at 32℃ at 1,000 - 2,000*g* to facilitate binding of virus particles with RetroNectin reagent.
3. Discard the supernatant, but do not allow the plate to dry. Wash the plate with an appropriate volume of PBS or PBS containing 0.1 - 2% albumin (BSA or HSA). Then perform virus infection according to A-3.

A-3. Virus Infection

Prepare the target cells while the retrovirus particles are binding to the RetroNectin reagent-coated plate. It is important that the target cells be in logarithmic growth phase and express integrin receptors VLA-4 and/or VLA-5. When using hematopoietic stem cells, pre-stimulation with cytokine may be necessary. The cytokine type should be determined based on your specific research protocols. Examples are cited in references 3 and 5.

1. Collect the target cells and count the number of living cells. Then suspend the cells in the growth medium at a concentration of 0.2 - 1 x 10^5 cells/ml.
2. Remove the wash solution from the virus-bound plates prepared by A-1 or A-2. Do not allow the plate to dry. Immediately add target cells at a density of 0.5 - 2.5 x 10^4 cells/cm^2. Although the optimal cell density depends on cell size and growth rate, the initial cell density should allow the cells to be actively growing or nearly confluent when analyzed 2 - 3 days after transduction. When infecting more cells, you may increase the cell density, but the cells will need to be subcultured after gene transduction.

   Note: To promote contact between the target cells and viral particles, plates can be centrifuged after adding the cells.
3. Incubate in a 37°C, 5% CO_2 incubator for 2 - 3 days.
4. Collect both non-adherent and adherent cells:
   (1) Transfer the supernatant to a centrifuge tube.
   (2) Recover remaining non-adherent cells by washing the plate with PBS.
   (3) Dissociate adherent cells from the plate with Cell Dissociation Buffer (Thermo Fisher Scientific), an enzyme-free solution, or trypsin-EDTA following the manufacturer's instructions.

   Note: For many cell types, adherent cells may be collected by pipetting only.
   (4) Combine the cells obtained from Steps (1) - (3) in the same tube, and centrifuge to recover the cells.
   (5) Wash the cells with HBSS/Hepes twice, collecting the cells by centrifugation. Suspend the cells in HBSS/Hepes* for further analysis.
   * Any buffer or medium suitable for downstream application of the cells can be also used for resuspension.

B. Supernatant (SN) Infection Method

When the virus stock solution is used, the RBV method described in A is recommended, but if a 4-fold dilution or more is used, either the RBV or SN method may be used, as an equivalent gene transduction efficiency will be obtained. The time required for virus infection is much shorter with the SN method than with the RBV method.

1. Suspend the target cells in virus solution that has been diluted with growth medium to prepare the cell suspension.
2. Add the cell suspension to the RetroNectin-coated plate at a density of $0.5 - 2.5 \times 10^4$ cells/cm^2. Although the optimal cell density depends on cell size and growth rate, the initial cell density should allow the cells to be actively growing or nearly confluent when gene expression is analyzed 2 - 3 days after transduction. When infecting more cells, you may increase the cell density, but the cells will need to be subcultured after gene transduction.

   Note: To promote contact between the target cells and virus vectors, the plate can be centrifuged after adding the cells.
3. Incubate in a 37°C, 5% CO_2 incubator for 2 - 3 days.
4. Collect both non-adherent and adherent cells:
   (1) Transfer the supernatant to a centrifuge tube.
   (2) Recover remaining non-adherent cells by washing the plate with PBS.
   (3) Dissociate adherent cells from the plate with Cell Dissociation Buffer (Thermo Fisher Scientific), an enzyme-free solution, or trypsin-EDTA following the manufacturer' s instructions.

   Note: For many cell types, adherent cells may be collected by pipetting only.
   (4) Combine the cells obtained from Steps (1) - (3) in the same tube, and centrifuge to recover the cells.
   (5) Wash the cells with HBSS/Hepes twice, collecting the cells by centrifugation. Suspend the cells in HBSS/Hepes* for further analysis.
   * Any buffer or medium suitable for downstream application of the cells can be also used for resuspension.

VI. References

1) Kimizuka F, Taguchi Y, Ohdate Y, Kawase Y, Shimojo T, Hashino K, Kato I, Sekiguchi K, and Titani K. Production and characterization of functional domains of human fibronectin expressed in *Escherichia coli. J Biochem*. (1991) **110**: 284-291.
2) Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, and Williams DA. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat Med*. (1996) **2**: 876-882.
3) Hanenberg H, Hashino K, Konishi H, Hock RA, Kato I, and Williams DA. Optimization of fibronectin-assisted retroviral gene transfer into human $CD34^+$ hematopoietic cells. *Hum Gene Ther*. (1997) **8**: 2193-2206.
4) Pollok KE, Hanenberg H, Noblitt TW, Schroeder WL, Kato I, Emanuel D, and Williams DA. High-efficiency gene transfer into normal and adenosine deaminase-deficient T lymphocytes is mediated by transduction on recombinant fibronectin fragments. *J Virol*. (1998) **72**: 4882-4892.
5) Chono H, Yoshioka H, Ueno M, and Kato I. Removal of inhibitory substance with recombinant fibronectin-CH-296 plates enhances the retroviral transduction efficiency of $CD34^+CD38^-$ bone marrow cells. *J Biochem*. (2001) **130**: 331-334.

【 Information: T-cell expansion by RetroNectin stimulation 】

RetroNectin reagent has been widely used to enhance retroviral and lentiviral gene transfer into mammalian cells.
In addition, RetroNectin reagent enhances the proliferation of T lymphocytes. T cells are conventionally expanded in the presence of Interleukin-2 (IL-2) by stimulation with anti-CD3 antibody. The addition of RetroNectin in this stimulation step dramatically increases the efficiency of T cell expansion. In addition, T cells expanded with this method contain a high proportion of less-differentiated T cells (naive T cells). Naive T cells have the ability to differentiate into cytotoxic T cells *in vivo*.

VII. Related Products

[Retroviral vectors]
pDON-5 Neo DNA (Cat. #3657)
pDON-5 DNA (Cat. #3658)
pDON-AI-2 Neo DNA (Cat. #3653)
pDON-AI-2 DNA (Cat. #3654)
pMEI-5 Neo DNA (Cat. #3655)
pMEI-5 DNA (Cat. #3656)

[Preparation of Recombinant Retroviral Particles]
Retrovirus Packaging Kit Ampho (Cat. #6161)*
Retrovirus Packaging Kit Eco (Cat. #6160)*
Retro-X™ System (Cat. #631508)
Retro-X™ Universal Packaging System (Cat. #631530)

[Lentiviral Vectors and Vector Systems]
Lenti-X™ Expression System (Cat. #632164)

[Preparation of Recombinant Lentiviral Particles]
Lenti-X™ 293T Cell Line (Cat. #632180)
Lenti-X™ Packaging Single Shots (VSV-G) (Cat. # 631275)
Lenti-X™ HTX Ecotropic Packaging System (Cat. #631251)

[Other]
RetroNectin® Dish (RetroNectin Pre-coated Dish, 35 mmφ) (Cat. #T110A)*
RetroNectin® GMP grade Recombinant Human Fibronectin Fragment CH-296 (Cat. #T201)*
Retro-X™ qRT-PCR Titration Kit (Cat. #631453)
Lenti-X™ qRT-PCR Titration Kit (Cat. #631235)

* Not available in all geographic locations. Check for availability in your region.

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